

APP EXHIBIT A

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.
8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength ($6 \times$ SSC or $6 \times$ SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, $6 \times$ SSPE is preferred because of its greater buffering power.
9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.
10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μ g of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/ μ g or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/ μ g or greater).
11. *Useful facts:*
 - a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5^\circ\text{C} + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\text{fraction G} + \text{C}) - 0.63(\% \text{ formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[\text{Na}^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) ($0.75^\circ\text{C}/1\%$ formamide) and less for DNAs rich in poly(dG:dC) ($0.50^\circ\text{C}/1\%$ formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257} . The "irreversible" T_m , which is more important for autoradiographic detection of DNA hybrids, is usually $7\text{--}10^\circ\text{C}$ higher than that predicted by the equation.

Similar equations have been derived for:

- i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8^\circ\text{C} + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\text{fraction G} + \text{C}) + 11.8(\text{fraction G} + \text{C})^2 - 0.35(\% \text{ formamide}) - (820/l)$$

- ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8^\circ\text{C} + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\text{fraction G} + \text{C}) + 11.8(\text{fraction G} + \text{C})^2 - 0.50(\% \text{ formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

- b. The T_m of a double-stranded DNA decreases by $1\text{--}1.5^\circ\text{C}$ with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).